TITLE. Toxicity of seabird guano to sea urchin embryos and interaction with Cu and Pb

AUTHOR NAMES Diego Rial[1]*, Juan Santos-Echeandía[2], Xosé Antón Álvarez-Salgado[2], Antoni Jordi[3], Antonio Tovar-Sánchez[3,4]. Juan Bellas[5].

AUTHORS ADDRESSES

[1] CIIMAR Ecotoxicology, stress ecology and environmental health, Rua dos Bragas 289, 4050-123 Porto, Portugal

[2] CSIC Instituto de Investigaciones Marinas, Eduardo Cabello 6, 36208 Vigo, Spain

[3] CSIC Instituto Mediterráneo de Estudios Avanzados −University of the Balearic Islands, Miguel Marqués 21, 07190 Esporles, Balearic Islands, Spain

[4] CSIC Instituto de Ciencias Marinas de Andalucía, Campus Universitario Río San Pedro, 11510 Puerto Real, Cádiz, Spain

[5] Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Vigo, Cabo Estai - Canido, 36200 Vigo, Spain

* Corresponding author e-mail: diegorialconde@gmail.com

CIIMAR Ecotoxicology, stress ecology and environmental health, Rua dos Bragas 289, 4050-123 Porto, Portugal

Abstract

Guano is an important source of marine-derived nutrients to seabird nesting areas. Seabirds usually present high levels of metals and other contaminants because the bioaccumulation processes and biotic depositions can increase the concentration of pollutants in the receiving environments. The objectives of this study were to investigate: the toxicity of seabird guano and the joint toxicity of guano, Cu and Pb by using the sea urchin embryo-larval bioassay. In a first experiment, aqueous extracts of guano were prepared at two loading rates (0.462 and 1.952 g L^{-1}) and toxicity to sea-urchin embryos was tested. Toxicity was low and not dependent of the load of guano used (EC_{50} 0.42±0.03 g L^{-1}). Trace metal concentrations were also low either in guano or in aqueous extracts of guano and the toxicity of extracts were apparently related to dissolved organic matter. In a second experiment, the toxicity of Cu-Pb mixtures in artificial seawater and in extracts of guano (at two loadings: 0.015 and 0.073 g L^{-1}), was tested. According to individual fittings, Cu added to extracts of guano showed less toxicity than when dissolved in artificial seawater. The response surfaces obtained for mixtures of Cu and Pb in artificial seawater, and in 0.015 g L^{-1} and 0.073 g L^{-1} of guano, were better described by Independent Action model adapted to describe antagonism, than by the other proposed models. This implied accepting that EC_{50} for Cu and Pb increased with the load of guano and with a greater interaction for Cu than for Pb.

Keywords. Antagonism; joint toxicity; response surface; sea urchin embryo; seabird guano; trace metals.

1. Introduction

Coastal areas and cliffs represent suitable areas for seabird nesting activities. Yellow-legged gull (*Larus michahellis*) is one of the main communities that forms these coastal colonies and tend to deposit large amounts of organic type material (faeces, feathers, corpses, foodstuff, etc.). Among these materials, faeces or guano represent around 85% of the dry weight (Wait et al., 2005). The guano is mainly rich in nutrients (N and P) so its inputs alter the surrounding environment. Several works have shown an increase in the nutrient content of soils (Bukacinski et al., 1994; Otero et al., 2015) but also in the adjacent coastal waters. This enrichment is associated to direct deposition (Kolb et al., 2010) or to rain and run-off events, sea spray or waves that wash guano from bird colonies into the sea. This may cause local nutrient enrichment (Bosman et al., 1986; Kolb et al., 2010) which may enhance marine phytoplankton production (Zelickman and Golovkin, 1972) and intertidal macroalgal growth (Bosman et al., 1986). It has been shown that the guano derived materials can reach several meters seawards (Kolb et al., 2010) .

In addition to the high nutrient contents, guano is also rich in organic matter, metals and other toxicants (Otero and Fernandez-Sanjurjo, 2000; Liu et al., 2006; Signa et al., 2013). The nitrogen content of seabird guano ranges from 8 to 21% of the fresh weight and consist mainly of uric acid (ca. 80%), proteins (ca. 10%), ammonia (ca. 7%) and nitrate (ca. 0.5%) (Szpak et al., 2012) . Concerning phosphorus, it represents from 0.1 to 10% of the fresh weight and consists of about 50 to 70% of phosphate (Smith and Johnson, 1995; Otero et al., 2015). Due to bioaccumulation, seabirds usually present high levels of metals and other contaminants, and their concentrations can increase in

the biotic depositions to toxic levels with negative implications for the ecosystems (Sun et al., 2000; Michelutti et al., 2010; Signa et al., 2013).

Due to the high content of organic material present in seabird guano, it may also represent an important input of dissolved organic matter to these coastal environments. The organic complexation of trace metals drives their biogeochemical cycles in coastal waters representing up to 99% of trace metal dissolved species (van den Berg et al., 1987; Saito and Moffett, 2001; Santos-Echeandia et al., 2008). This binding reduces metal lability and toxicity for marine organisms. Thus, it may be expected that this extra input of organic matter could be a natural defence for coastal littoral species against punctual inputs of trace metals as it has been demonstrated with other sources of organic matter (i.e. sediments or sewage) (Sanchez-Marin et al., 2010).

Due to their environmental relevance, metals have received particular attention in toxicity studies with aquatic organisms, but focus has been mostly placed on individual toxicity. However, aquatic organisms are usually exposed simultaneously to a wide range of toxicants in the environment rather than to individual substances. Thus, the inclusion of the combined effects of toxicants resulting from multiple exposures in water quality regulations has been advocated (Bellas, 2008). Although, the experimental evaluation of all possible combinations of toxicants is not feasible, several models have been developed for the prediction of combination effects on the basis of the concentration-response relationships of individual mixture components. Mixture toxicity studies conducted with aquatic organisms have reported either antagonistic, additive or synergistic interactions, depending on the metals studied and on the test species (Meyer et al., 2015).

It is generally accepted that embryos and larvae are the most sensitive developmental stages in the life cycle of marine invertebrates. Because of their high sensitivity, rapid response and ecological relevance, the embryo-larval bioassays with marine invertebrates, in particular with bivalves and sea urchins, have been used for decades in the toxicity evaluation of marine pollutants (Kobayashi, 1995; His et al., 2000). The sea urchin embryo test has also been used to investigate the toxicity of complex matrices as sediment or petroleum using different methods (porewater, elutriate, water accommodated fraction, chemical extraction…) (Losso et al., 2009; Beiras et al., 2012; Rial et al., 2013). We report here toxicity tests with the edible sea-urchin *Paracentrotus lividus* (Lamarck, 1816), a large regular sea-urchin widely distributed throughout the Mediterranean Sea and European Atlantic coast with important ecological roles in the functioning, dynamics and structure of benthic assemblages (Hayward and Ryland, 1990; Boudouresque and Verlaque, 2007). Also, several studies have shown the importance of sea-urchin pluteus larvae in the composition and biomass of zooplankton communities, playing a significant role in the pelagic food web (Luis et al., 2005). In some European countries *P. lividus* is also exploited for its highly valued gonads (Boudouresque and Verlaque, 2007).

The objectives of this study were to investigate: 1) the toxicity of seabird guano for sea-urchin embryos; and 2) the joint toxicity of two metals, Cu and Pb, in presence of seabird guano as a source of organic matter.

2. Material and methods

Around 60 plastic collectors were placed at the Islote dels Conills (Parque Nacional Marítimo-Terrestre del Archipiélago de Cabrera, Balearic Islands) in order to get the guano samples. A representative colony of yellow-legged gull (*Larus michahellis*), the most representative seabird in the archipelago, inhabits this small island. Guano samples were collected during 2013-2014 making a unique and homogenized sample. Samples were frozen at -20ºC just after collection until experiments were conducted.

Once in the lab, samples were freeze-dried, ground and preserved in plastic vials waiting for its chemical characterization and experiments.

2.1. Chemical characterization of the original guano samples

Metal determinations were carried out in the guano. Prior to analysis samples were microwave-digested (Milestone 1200 Mega, Milestone Inc., USA) in Teflon bombs using a mixture of HNO_3 and HF according to EPA guideline 3052 (USEPA, 1996).

According to the content level of metal in the samples, analyses were conducted using Electrothermal Atomic Absorption Spectrometry (Varian 220, Varian Inc., USA) equipped with Zeeman background correction for the determination of Cd, Co, Cr, Cu, Ni and Pb. On the other hand, Al, Fe and Zn were measured with Flame-Atomic Absorption Spectrophotometry (Varian SpectrAA 220FS). The accuracy of the analytical procedure was checked using the reference material PACS-2 (marine sediment reference material) and was in good agreement with the certified values. For all analysed metals, the recoveries were in the range of 94% to 103%.

Particulate organic carbon and nitrogen content of the freeze-dried guano sample was determined by high temperature (900ºC) catalytic oxidation in an Elemental Analyser Perkin Elmer 2400 (PerkinElmer Inc., USA).

2.2. Aqueous extracts of guano

Different proportions of guano:water were applied in order to discriminate if the load of guano determined the amount of dissolved substances in the aqueous extract and their toxicity (subsection 2.2.1).

The evaluation of the joint toxicity of Cu, Pb and guano required an experimental design adequate to describe the interactions of metals and the effect of the organic matter released from guano on their toxicity (subsection 2.2.2).

2.2.1. Loading rate test

Loading rate is defined as the ratio of guano to water used in the preparation (OECD, 2000). Aqueous extracts of guano were prepared at two loadings by adding 0.231 and 0.976 g of guano to 500 mL of artificial seawater (ASW) (0.462 and 1.952 g L^{-1} respectively). Subsequently, extracts were exposed to an ultrasonic bath for 30 minutes, to rotatory mixing for 30 min and finally to overnight decantation. The aqueous extract was filtered through a 0.45 μm polyethersulfone filter (Pall corp., USA) in order to remove particulate material and was diluted with ASW (0, 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 mL L^{-1}). Diluted samples were used concurrently for chemical analyses and for toxicity tests.

2.2.2. Mixtures of Cu and Pb at different concentrations of guano

Aqueous extracts of freeze-dried guano were performed in triplicate by adding 0.181 g to 500 mL of ASW using the procedure mentioned above. The aqueous extracts were siphoned, filtered through a 0.45 μm polyethersulfone filter and diluted with ASW to final concentrations of 0 mL L^{-1}, 40 mL L^{-1} (0.015 g/L) and 200 mL L^{-1} (0.073 g L^{-1}) in 2 L clean acid plastic bottles.

20 mL of each diluted extracts (0, 40 and 200 mL L^{-1}) and 100 μL of a stock solution of Cu and/or Pb were added to 25 mL plastic vials. The resultant nominal concentrations were 0, 8, 12, 16, 20, 24, 30, 38, 50 and 100 μg L^{-1} for Cu and 12.5, 25, 50, 100, 130, 175, 250, 500 and 1000 μg L^{-1} for Pb. Mixtures corresponding to 4x4 combinations of Cu (12, 16, 24 and 38 μg L^{-1}) and Pb (25, 50, 130 and 250 μg L^{-1}) were also performed. Solutions were kept for 1 h in orbital shaking at 150 rpm to allow equilibration of the complexation reaction.

2.2.3. Chemical characterization of the elutriates and metal solutions

Samples for the analysis of inorganic nutrients were collected in acid-cleaned 50-mL polyethylene bottles; they were frozen at -20ºC until determination using standard segmented flow analysis with colorimetric detection in an Alliance Futura analyser (AMS S.p.A, Italy). The precisions were ±0.02 μM for nitrite and phosphate, ±0.1 μM for nitrate and ±0.05 μM for ammonium and silicate.

For the analysis of dissolved organic carbon (DOC), samples were taken in acid-cleaned 20-mL amber glass flasks with Teflon caps. After acidification with H_3PO_4 to pH <2, the flasks were capped and stored in the dark at 4 ºC until analysis. DOC was measured

189 by high temperature (680 ºC) catalytic oxidation with a Shimadzu TOC-V organic
190 carbon analyser (Shimadzu corp., Japan). The precision of the method was ± 1 μM.
191
192 Dissolved trace metal analyses were conducted for elutriates and selected solutions of
193 Cu and Pb by means of stripping voltammetry using a Metrohm797 VA computrace
194 equipped with a hanging mercury drop electrode as the working electrode, Ag/AgCl as
195 the reference electrode, and a Pt wire as the counter-electrode. Prior to determination,
196 samples were UV-digested for 1 h using an UV-Digestor equipped with a high-pressure
197 mercury lamp of 200 W (Achterberg and van den Berg, 1994). The simultaneous
198 determination of Cd, Cu and Pb in the dissolved phase was carried out using the method
199 of standard additions by differential pulse anodic stripping voltammetry (DPASV)
200 (Gardiner and Stiff, 1975) while the simultaneous determination of Co and Ni was
201 performed by Adsorptive Cathodic Stripping Voltammetry (ACSV) (Santos-Echeandia,
202 2011). The solution was deaerated by purging (5 min) with nitrogen. Voltammetric
203 parameters for the DPASV method were: deposition 300–900 s at −1.1 V whilst
204 stirring; 10 s quiescence at −1.1 V; potential scan using the differential pulse
205 modulation: pulse amplitude of 50 mV, a pulse duration of 40 ms, a pulse frequency of
206 5 s^{-1} and a scan rate of 20 mV s^{-1}, from −1.1 to 0 V. In the case of the ACSV method,
207 voltammetric parameter were: deposition 30–120 s at −0.35 V whilst stirring; 10 s
208 quiescence at −0.05 V; potential scan using the differential pulse modulation: pulse
209 amplitude of 50 mV, a pulse duration of 40 ms, a pulse frequency of 5 s^{-1} and a scan
210 rate of 20 mV s^{-1}, from −0.05 V to −1.2 V.
211

The accuracy of the analytical procedure was assessed by the analysis of two different certified reference materials (CASS-4 and SLEW-3), obtaining good agreement with the certified concentrations. Recoveries for all the elements were between 96-102%.

2.3. Sea urchin embryo test

The sea urchin embryo test was performed in accordance with the method of Saco-Álvarez et al.(2010). Gametes of *Paracentrotus lividus* were obtained by dissection of two adult sea urchins and their maturity (ovum sphericity and sperm mobility) checked with a microscope. The ova were transferred to a 100 mL graduated cylinder containing ASW (5-10 ova μL^{-1}). Then a few drops of sperm (30-100 μL), taken from the male gonad, were added, and the mixture was shaken gently to facilitate fertilisation. The fertilisation rate was determined in a Sedgewick-Rafter counting chamber in quadruplicate (n=100), as the proportion of eggs with a fertilisation membrane (control fertilisation success was always > 97%). Within 30 minutes, the fertilised eggs were transferred to vials with 4 mL of ASW containing the experimental solutions. Each vial received 40 eggs per mL and each treatment was performed in quadruplicate.

Eggs were incubated in the dark at 20 ºC for 48 hours, until larvae reached the four-arm pluteus stage. After the incubation period larvae were fixed and preserved by adding a few drops of 40% formalin. In each vial the maximum length of 35 individuals was measured using an inverted microscope and Leica QWIN image analysis software, version 3.4.0 (Leica Microsystems, Germany). The inhibition of growth in length was quantified as:

$$R_i = 1 - \frac{\Delta L_i}{\Delta L_0} \quad (1)$$

where ΔL_0 and ΔL_i are the mean length increases in control and in the i^{th} dose, respectively.

2.4. Mathematical models

The cumulative function of the Weibull distribution was used as a dose-response model (denoted by ${}^{m}W$):

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{C}{m}\right)^{a}\right]\right\}; \text{ or briefly: } R = {}^{m}W\left(C; K, m, a\right) \quad (2)$$

where R is the response (with K as maximum value), C is the concentration, m is the dose corresponding to the semi-maximum response and a is a shape parameter related to the maximum slope of the response.

Equation 2 can be re-parameterized to estimate the slope at the median abscissa of Weibull density function:

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{C}{Ka\ln 2 / 2v_{med}}\right)^{a}\right]\right\} \quad (3)$$

A four-parameter model can also be used for those data sets in which a response higher than 0 is observed for the control (R_c):

$$R = R_c + K\left\{1 - \exp\left[\ln 2\left(\frac{C}{m}\right)^a\right]\right\} \quad (4)$$

To directly obtain the confidence intervals of the EC_{50}, equation 4 was re-parameterized to make explicit the corresponding dose:

$$R = R_c + K\left\{1 - \exp\left[\ln\left(1 - \frac{0.5 - R_c}{K}\right)\left(\frac{C}{EC_{50}}\right)^a\right]\right\} \quad (5)$$

2.4.1. CA hypothesis

The inverse of equation 2 is required to apply the Concentration Addition (CA) model:

$$C_i = f_i^{-1}(R) \text{ or } C_i = m_i\left[\frac{\ln\left(1 - \frac{R}{K_i}\right)}{-\ln 2}\right]^{1/a_i} \quad (6)$$

where C_i is the concentration of the effector *i* that produces the response *R* and $f^{-1}(R)$ is the inverse function of the Weibull model. The CA model has to be solved by iteration to find the parameters of the single equations and the values of the predicted response (*R*) which minimizes the residual sum of squares and satisfy the following condition (Berenbaum, 1985):

$$\sum_{i=1}^{n}\frac{c_i}{f_i^{-1}(R)}=1;\ \text{or more commonly}\ \sum_{i=1}^{n}\frac{c_i}{C_i}=1 \qquad (7)$$

271

272 where, c_i is the concentration of chemical *i* in the mixture and C_i is given by expression

273 (6).

274

275 A unique maximum response (*K*) is assumed in the CA model (Jonker et al., 2005). The

276 equation for three toxic agents is as follows:

277

$$\frac{c_1}{f_1^{-1}(R)}+\frac{c_2}{f_2^{-1}(R)}+\frac{c_3}{f_3^{-1}(R)}= \qquad (8)$$

$$\frac{c_1}{m_1\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_1}}+\frac{c_2}{m_2\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_2}}+\frac{c_3}{m_3\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_3}}=1$$

278

279 A simplified version of the CA model (hereinafter called CA_M), which makes possible

280 to obtain directly the function parameters by non linear regression, reported by Murado

281 and Prieto (2013), was adapted here to three effectors:

282

$$R=W\left[\left(C_1+u_2C_2+u_3C_3\right);K,m,a\right] \qquad (9)$$

283

284 where C_1, C_2 and C_3 are the concentrations of the effector 1, 2 and 3, respectively, and

285 u_2 and u_3 are the factors that show the relative toxic potency of effectors 2 and 3

regarding to effector 1. The same maximum effect (K) and shape parameter (a) is assumed for all effectors.

Murado and Prieto (2013) have also proposed a modification of the CA model to describe synergism or antagonism. In that model it is assumed that a toxic agent can increase or decrease the effective dose of other effector. That model was adapted here to three effectors and to a particular condition: an unidirectional interaction of the toxic agent 3 (guano as source of organic matter) over the effective concentrations of agents 1 (Cu) and 2 (Pb) (hereinafter called CA_SA):

$$R = W\left[\left(C_1\pi_{C1} + u_2 C_2 \pi_{C2} + u_3 C_3\right); K, m, a\right] \qquad (10)$$

$$\pi_{C1} = \left(1 + b_{c1} C_3\right) / \left(1 + c_{c1} C_3\right);\ \pi_{C2} = \left(1 + b_{c2} C_3\right) / \left(1 + c_{c2} C_3\right)$$

2.4.2. IA hypothesis

The independent action (IA) model for three agents is formulated as follows (Bliss, 1939):

$$R(C_{\text{mix}}) = 1 - \left[1 - R(C_1)\right]\left[1 - R(C_2)\right]\left[1 - R(C_3)\right] = \qquad (11)$$

$$= 1 - \left[1 - W(C_1; K_1, m_1, a_1)\right]\left[1 - W(C_2; K_2, m_2, a_2)\right]\left[1 - W(C_3; K_3, m_3, a_3)\right]$$

where $R(C_{\text{mix}})$ is the response corresponding to the mixture, and $R(C_1)$, $R(C_2)$and $R(C_3)$ are the response to the agents 1, 2 and 3.

Murado and Prieto (2013) have also proposed a modification of the IA model to describe synergy or antagonism, in which it is assumed that the concentration of each toxic agent may alter the parameters value of the toxicity model of the other agents considered. This model is adapted here to three effectors and an unidirectional interaction of an agent over the other two effectors (IA_SA):

$$R = 1 - \left[1 - W\left(C_1; K_1\pi_{K1}, m_1\pi_{m1}, a_1\pi_{a1}\right)\right]\left[1 - W\left(C_2; K_2\pi_{K2}, m_2\pi_{m2}, a_2\pi_{a2}\right)\right] \left[1 - W\left(C_3; K_3, m_3, a_3\right)\right] \quad (12)$$

$$\pi_{\theta 1} = \left(1 + b_{\theta 1}C_3\right)/\left(1 + c_{\theta 1}C_3\right);\ \pi_{\theta 2} = \left(1 + b_{\theta 2}C_3\right)/\left(1 + c_{\theta 2}C_3\right);\ \theta = K, m, a$$

2.5. Statistical analyses

Fitting procedures and parametric estimations from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro '*Solver*' of the *Microsoft Excel* spreadsheet. Confidence intervals from the parametric estimations (Student's *t* test) were determined with the freely available '*SolverAid*' macro and the consistence of mathematical models assessed by a Fisher's *F* test.

The Akaike´s information criterion (AIC) was used for comparing models. AIC statistics summarize goodness-of-fit as residual sum of squares (*RSS*) against the number of parameters (*p*) for the same data set (*n*) with the aim of avoiding over-fitting. AIC can be defined as (Motulsky and Christopoulos, 2003):

$$AIC = n\ln\left(\frac{RSS}{n}\right) + 2(p+1) + \left[\frac{2(p+1)(p+2)}{n-p-2}\right] \quad (13)$$

The model with the lowest AIC is the one with the highest likelihood of being correct.

3. Results

3.1. Chemical characterization of the original guano samples

The reason for analysing the chemical composition of guano was to check for the potential toxicity of some of its elements. Trace metal concentrations in guano were: 12.7±0.20 mg g^{-1} for Al, 0.55±0.05 µg g^{-1} for Cd, 3.62±0.09 µg g^{-1} for Co, 19.1±1.51 µg g^{-1} for Cr, 21.6±0.6 µg g^{-1} for Cu, 5.21±0.39 µg g^{-1} for Fe, 6.29±0.62 µg g^{-1} for Ni, 8.30±0.01 µg g^{-1} for Pb and 154±10 µg g^{-1} for Zn.

Regarding carbon and nitrogen composition of guano, 26.4±0.2% (w/w) was C and 7.26±0.12% was N.

3.2. Composition of aqueous extracts of guano

In order to check the solubility of trace metals, these elements were analysed in the elutriates. Results are shown in Table 1 together with nutrients and dissolved organic carbon concentrations.

3.3. Loading rate test

The inhibition of sea urchin larval growth exhibited a similar pattern for the extracts of guano obtained at different loadings (Figure 1, left) and the parameters EC_{50}, EC_{10} and *a* of the Weibull model showed overlapping confidence intervals (Table 2). An F-test was used to assess whether a single model (null hypothesis) or two separate curves for

each load described better the data (alternative hypothesis) and it was accepted the null hypothesis (p=0.093).

The dissolved organic carbon (Figure 1, right) may contribute to the observed toxicity for the extract of guano (Figure 1, left). How6ever, the parameter m (g L^{-1}) for the inhibitory response (0.37±0.03) (Figure 1, left) was less than that of DOC (0.59±0.07) (Figure 1, right), and the relative slope (v_m/K) was markedly greater for toxicity (1.3) than for DOC (0.8). So other factors may have contributed to the toxicity of guano.

The drop in pH value for undiluted extracts of low (8.02) or high load (7.52), with respect to ASW (8.11), does not serve to explain an increase in toxicity due to this parameter (Saco-Álvarez et al., 2010). The NH_3 concentrations calculated for dilutions of the extracts in the treatment of low (1.0-39.4 μg L^{-1}) or high load (0.3-31.7 μg L^{-1}) did not explain the observed toxicity either (Saco-Álvarez et al., 2010).

The concentrations of metals measured in undiluted extracts of low and high loads were not relevant from a toxicological point of view except for Cu (Table 1). For Cu, 0.2 toxic units were calculated in the undiluted extracts of both low and high loads.

3.4. Mixtures of Cu and Pb at different concentrations of guano

A good agreement was found between nominal and measured concentrations (in brackets) for Cu 12 (11.8±0.2) μg L^{-1}, 24 (23.8±0.1) μg L^{-1} and 50 (49.7±0.1) μg L^{-1} and Pb 25 (24.4±0.4) μg L^{-1}, 130 (129.6±0.3) μg L^{-1} and 500 (499.2±0.1) μg L^{-1} in ASW.

375 Cu toxicity in ASW (EC_{50} = 33.09 μg L^{-1} or 0.52 μM) was greater than that of Pb
376 (355.87 μg L^{-1} or 1.72 μM) (Table 3). The slope of the curve (v_m) was also significantly
377 higher for Cu (0.0192 L μg^{-1} or 1.22 μM^{-1}) than for Pb (0.0010 L μg^{-1} or 0.21 μM^{-1})
378 (Table 3).
379
380 The extracts of guano presented moderate toxicity in the absence of added Cu or Pb,
381 showing inhibitory responses (R) of 0.18 and 0.30 for the low and high concentration
382 respectively (Figure 2). Metal concentrations measured in the extracts of guano did not
383 serve to explain the observed toxicity (Table 1), since toxic units could only be
384 calculated for Cu and the values were quite low (0.1 and 0.2 for low and high
385 concentration respectively).
386
387 Cu added to extracts of guano showed less toxicity than Cu dissolved in ASW. Figure 2
388 shows how guano inhibits sea-urchin growth at low concentrations of Cu and reduces
389 Cu toxicity at higher concentrations. A reduction of Cu toxicity was observed in terms
390 of both EC_{50} and slope (v_m) for the concentrated extract of guano and a lower value of
391 v_m was also found for the diluted extract (Table 3 and Figure 2). For Pb, unlike Cu,
392 significant differences were only found in terms of v_m (Table 3 and Figure 2). Lower
393 EC_{50} values for Pb were found in guano treatments than in ASW, though these values
394 did not differ significantly (Table 3). However, the comparison of the EC_{50} values is not
395 straightforward, due to the intrinsic toxicity of guano in the absence of added metals and
396 the impossibility of separating its inhibitory effects of the considered metal (Figure 2).
397
398 The model of Independent Action (IA) for two effectors (AIC = -286.82, adj. R^2 =
399 0.978) described better the joint toxicity of Cu and Pb in ASW than Concentration

400 Addition (CA) (AIC = -275.9, adj. R^2 = 0.972) or Concentration Addition Modified
401 (CA_M) (AIC = -249.34, adj. R^2 = 0.948).
402
403 The joint inhibition of Cu, Pb and extract of guano was well described by the model
404 IA_M_SA (Figure 3). The lowest value of the Akaike Information Criterion and the
405 maximum value of adjusted R^2 were obtained for this model (Table 4). The parameters
406 of the model were significant for Cu and Pb, but *K* for guano was not significant (Table
407 4). The model IA_M_SA implies accepting that *m* for Cu or Pb increases with the
408 concentration of guano according to the expression (12), and the following values of b_{m1}
409 = 11.580 (interaction parameter of guano on *m* of Cu) and b_{m2} = 5.974 (interaction
410 parameter on *m* of Pb) were obtained (Table 4). The value of b_{m1} was approximately
411 double that of b_{m2}, indicating a greater interaction of guano for Cu than for Pb. A similar
412 description and identical values of b_{m1} and b_{m2} was obtained using model 3 as the core
413 of model 12.
414
415 **4. Discussion**
416 Trace metal concentrations in guano were low. In fact, our values were lower than those
417 determined by Otero (1998) in faeces collected from fishing ports of Galicia for the
418 same species and which could be related to the low concentrations of heavy metals –
419 except mercury– measured in sediments from archipelago of Cabrera (Tovar-Sánchez et
420 al., 2011). Concentrations of Cd, Cr, Cu, Ni and Pb were lower than the Effects Range
421 Low (ERL), indicative of concentrations below which adverse effects in marine
422 sediments rarely occur, although Zn showed a slightly higher value (154 µg g^{-1}) than of
423 this guideline (150 µg g^{-1}) (Long et al., 1995).
424

Guano showed low toxicity to sea urchin embryos and its toxicity seems to be related in part to the dissolved organic matter released from the guano. The two treatments assayed in the loading rate test were homogeneous in terms of growth inhibition (Figure 1, left) and the dissolution of organic carbon in seawater showed a pattern which is apparently independent from the load of guano used (Figure 1, right). The toxic contribution of NH_3 and metals in the extracts of guano was low, but it cannot be ruled out that other unmeasured toxicants may have contributed to the growth inhibition observed in the sea urchin test. *NOEC* and EC_{10} for unionized ammonia were 40 and 68.4 μg L^{-1} according to Saco-Álvarez et al. (2010) and the unionized ammonia measured in the extracts were 0.3-39.4 μg L^{-1} for the loading rate test and 3.8-13.3 μg L^{-1} for the mixture toxicity test; so unionized ammonia did not explain the observed toxicity. A slight drop in pH was observed in the loading rate test for undiluted extracts of guano (pH: 7.52-8.02) compared to control (8.11), which may be due to the acids contained in guano (mainly uric acid). However, these values are well above the *NOEC* (pH = 7) for the sea urchin test (Saco-Álvarez et al., 2010). The concentrations of DOC measured in the mixture toxicity test were 149 and 375 μM-C for low (0.015 g L^{-1}) and high load (0.073 g L^{-1}) of guano respectively. These values were comparatively greater than those obtained in the loading rate test (Table 1 and Figure 1). When the ratio DOC/load of guano was calculated, a higher value was shown for the mixture toxicity test (5163-10297 μmol-C g_{guano}^{-1}) than for the loading rate test (3167-5367 μmol-C g_{guano}^{-1}), which might be related to the use of a lyophilized powder of guano instead of dry guano in the former. The toxicity of guano in the mixture toxicity test (lyophilized) was also considerably higher than that of the loading rate test (dry), but it was not explained by the increase of solubilization. It was not possible to achieve a convincing interpretation for the difference in the toxicity values obtained in both tests, although

two explanations might be given: a) an increase of colloidal concentration in the mixture toxicity test associated to a higher toxicity, or b) different ratio of Dissolved Organic Carbon/Dissolved Organic Matter for the two tests.

The EC_{50} of Cu (33.1 μg L^{-1}) was significantly higher than that of Pb (355.9 μg L^{-1}), and is indicative of different mechanisms of toxicity or affinity for specific receptors. These values are similar to those found by Lorenzo et al. (2006) (35.6 μg L^{-1}) and Sánchez-Marín et al. (2010) (22.2 μg L^{-1}) for Cu, and by Sánchez-Marín et al. (2010) (406.1 μg L^{-1}) for Pb. The difference in slope (v_m) between Cu (0.0192 L μg^{-1}) and Pb (0.0010 L μg^{-1}) is even higher than for EC_{50} values. It is well known that metals can react with enzymes, cell membranes and specific cellular components. Tellis et al. (2014a, b) have pointed out that both Cu and Pb impair the function of the ionic regulation (especially Ca homeostasis), interact with sulfhydryl groups of enzymes and generate free radicals in sea-urchin larvae. However, Radenac et al. (2001) showed that although Pb was accumulated more than Cu in *P. lividus* larvae, its toxicity was much lower than that of Cu, which suggested different mechanisms of toxicity for both metals.

The joint toxicity of Cu and Pb is determined by their specific mechanisms of toxicity. The observed response for binary mixtures of Cu and Pb in ASW was better described by IA (11) than by CA (8) or CA_M (9) models. Either of the reference models -CA or IA- allow to predict the joint toxicity of a mixture but with different assumptions: CA assumes that the components of the mixture present the same or similar mode of action while diverse or "dissimilar" modes of action are expected with the IA model (Kortenkamp and Altenburger, 2010). Thus, dissimilar modes of action are presumed

for Cu and Pb according to our results. It should be noted that CA described significantly better the observed response than CA_M; this latter model is a simplification of the CA that assumes equal curve shape but different toxic potency between Cu and Pb, which could not be accepted in this case. Xu et al. (2011) also evaluated the joint toxicity of Cu and Pb for embryos of the sea-urchin *Strongylocentyotus intermedius* and reported a weak antagonistic effect for these mixtures on the basis of the CA model. Some authors have pointed out the need to predict the toxicity of mixtures of metals in a more accurate way (e.g. (Meyer et al., 2015), but it would be necessary to emphasize that the interpretation of the results is conditioned by the experimental design previously performed. The experimental design used here allowed to perform an adequate description of the results and choose unambiguously between the reference models proposed.

The model used to describe the joint toxicity of Cu, Pb and guano (12) shows the particularity of including the guano as a source of organic matter and as an additional toxicant. The inhibitory response of guano in absence of Cu and Pb at low (0.18) and high (0.30) concentration can be seen in Figure 2. Hence, a marked toxic contribution of guano in the treatments of guano and metal cannot be discarded at a response level of 0.5, which prevents a direct comparison of EC_{50} values for the three treatments shown in Figure 2. In this figure it can be seen clearly how guano inhibits sea-urchin larval growth at low concentrations of Cu and reduces Cu toxicity at higher concentrations. It is known that dissolved organic matter from different origins may cause dose-dependent inhibition effects in the sea-urchin embryo development (Sanchez-Marin et al., 2010; Nadella et al., 2013), which justifies its inclusion in the model of joint toxicity. This allowed us to infer the inhibitory effects of guano, metals and their interaction.

The interaction described by model (12) involves an increase in m (EC_{50}) values, for both Cu and Pb, with increasing concentrations of guano (Figure 3). The magnitude of the interaction is greater for Cu (b_{m1} = 11.580) than for Pb (b_{m2} = 5.974), which indicates a higher affinity of Cu than Pb for dissolved organic matter from guano. A decrease in Cu toxicity to *P. lividus* larvae in the presence of organic matter from different origins, including humic and fulvic acids, has been previously reported (Lorenzo et al., 2002, 2006; Sanchez-Marin et al., 2010). However, the interaction of dissolved organic matter with Pb is less clear: increased toxicity with humic and fulvic acids (Sanchez-Marin et al., 2010; Sánchez-Marín and Beiras, 2012) and different effects depending on the type of organic matter tested (Sanchez-Marin et al., 2010). Sánchez-Marín et al. (2010) also reported that the complexing capacity of dissolved organic matter from different origins was lower in all cases for Pb than for Cu, which is consistent with the values found here for b_{m1} and b_{m2}.

Dissolved organic matter from guano differs from other sources, such as humic or fulvic acids, since it presents a lower heterogeneity of groups that can act as ligands of metal cations. Birds excrete nitrogenous waste primarily as uric acid and some of this uric acid is degraded by bacteria; although the proportions may vary depending on the species and diet (Lindeboom, 1984; Fugler, 1985). Proteins are also excreted by seabirds in lower proportions (Szpak et al., 2012). The major form of excreted phosphorus in guano is phosphate but only a reduced proportion is soluble (Smith and Johnson, 1995; Otero et al., 2015). Therefore, major chemical compounds present in the extracts of guano are expected to be uric acid, proteins, ammonia and phosphate (Smith and Johnson, 1995), so it is likely that the drop in toxicity observed for Cu and Pb may

525 be due to complexation or precipitation by these species. Hydrogen urate is the
526 predominant form of uric acid at pH = 8 and it has complexing capacity on Cu or Pb,
527 forming a non-electrolitic complex due to charge neutralization of the cation (Cu^{+2} or
528 Pb^{+2}) (Wilcox et al., 1972; Tak et al., 1981; Moawad, 2002). The solubility product
529 constants (K_{sp}) of the hydrogen urates were 5.8×10^{-5} for Cu (Moawad, 2002) and $1.2 \times$
530 10^{-14} for Pb (Tak et al., 1981). Hydrogen phosphate is the predominant phosphate
531 species at pH = 8 and shows K_{sp} values at 25 ºC of 4.5×10^{-7} for Cu and 5.3×10^{-12} for
532 Pb (Markich et al., 2001).. Therefore, according to these values, precipitation of Pb is
533 possible by either hydrogen phosphate or hydrogen urate but is unlikely for Cu.
534 Therefore, the reduction of the Cu toxicity is possibly due to the complexing capacity of
535 other dissolved species in the extract of guano such as protein materials given the
536 recognised capability of amino acids to bind with Cu and Pb (Sovago et al., 1993).
537
538 The guano is an important source of marine-derived nutrients in seabird breeding
539 islands, but shows little relevance in other coastal areas regarding alternative sources of
540 nutrients (Bedard et al., 1980; Bosman et al., 1986). Wootton (1991) found that guano
541 had a positive influence on 4 of 18 taxonomic groups from intertidal communities on
542 cliffs and led to a reduction of biomass in some groups which could have been caused
543 by ammonia from guano. The results of this study do not point in that direction as the
544 observed toxicity appeared to be related to the dissolved organic matter or other
545 unmeasured toxic agents.
546
547 **5. Conclusions**
548 The toxicity of guano to sea urchin embryos has been tested at environmentally relevant
549 concentrations. Low toxicity of guano was found which was apparently related to the

dissolved organic matter and independent from the load of guano used. The compounds dissolved from guano diminished both Cu and Pb toxicity. The response surfaces obtained for mixtures of Cu and Pb in artificial seawater, low and high load of guano were better described by Independent Action model adapted to describe antagonism than by the other proposed models. The magnitude of the interaction of guano was greater for Cu than for Pb, which indicated a higher affinity of the former for the dissolved organic matter from guano than the latter.

Acknowledgements

This work was partly supported by the Spanish Ministry of Agriculture, Food and Environment Grant PN384/2011. Diego Rial was funded by a postdoctoral contract from the Xunta de Galicia (ED481B 2014/098-0 Plan I2C), Spain (Plan I2C, 2014). The work of A.J. was supported by a Ramón y Cajal grant from the Spanish Ministry of Economy and Competitiveness. The CSIC, under the program JAE (Junta para la Ampliación de Estudios) co-funded by the Fondo Social Europeo (FSE), is greatly acknowledge for the post-doctoral fellowships to J. Santos-Echeandía.

References

Achterberg, E.P., van den Berg, C.M.G., 1994. In-line ultraviolet-digestion of natural-water samples for trace-metal determination using an automated voltammetric system. Anal.Chim.Acta 291, 213-232.

Bedard, J., Therriault, J.C., Berube, J., 1980. Assessment of the importance of nutrient recycling by seabirds in the St-Lawrence estuary. Can.J.Fish.Aquat.Sci. 37, 583-588.

573 Beiras, R., Durán, I., Bellas, J., Sánchez-Marín, P., 2012. Biological effects of
574 contaminants: *Paracentrotus lividus* sea urchin embryo test with marine sediment
575 elutriates. ICES Tech.Mar.Environ.Sci. 51, 13.
576 Bellas, J., 2008. Prediction and assessment of mixture toxicity of compounds in
577 antifouling paints using the sea-urchin embryo-larval bioassay. Aquat.Toxicol. 88, 308-
578 315.
579 Berenbaum, M.C., 1985. The expected effect of a combination of agents: the general
580 solution. J.Theor.Biol. 114, 413-431.
581 Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann.Appl.Biol. 26, 585-615.
582 Bosman, A.L., Du Toit, J.T., Hockey, P.A.R., Branch, G.M., 1986. A field experiment
583 demonstrating the influence of seabird guano on intertidal primary production.
584 Estuar.Coast.Shelf S. 23, 283-294.
585 Boudouresque, C.F., Verlaque, M., 2007. Ecology of *Paracentrotus lividus*. Elsevier
586 publ., Amsterdam, The Netherlands.
587 Bukacinski, D., Rutkowska, A., Bukacinska, M., 1994. The effect of nesting black-
588 headed gulls (*Larus ridibundus* L.) on the soil and vegetation of a vistula river island,
589 Poland. Ann.Bot.Fenn. 31, 233-243.
590 Fugler, S., 1985. Chemical composition of guano of burrowing petrel chicks
591 (Procellariidae) at Marion Island. Antarctic nutrient cycles and food webs. Springer, pp.
592 169-172.
593 Gardiner, J., Stiff, M.J., 1975. Determination of cadmium, lead, copper and zinc in
594 ground-water, estuarine water, sewage and sewage effluent by anodic-stripping
595 voltammetry. Water Res. 9, 517-523.
596 Hayward, P.J., Ryland, J.S., 1990. The Marine fauna of the British Isles and North-West
597 Europe. Clarendon Press; Oxford University Press, Oxford.

His, E., Beiras, R., Seaman, M.N.L., 2000. The assessment of marine pollution - Bioassays with bivalve embryos and larvae. Adv.Mar.Biol. 37, 1-178.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ.Toxicol.Chem. 24, 2701–2713.

Kobayashi, N., 1995. Bioassay for marine pollution using echinoderms. Encyclopedia of Environmental Control Technology, Vol 9, pp. 539-609.

Kolb, G.S., Ekholm, J., Hambäck, P.A., 2010. Effects of seabird nesting colonies on algae and aquatic invertebrates in coastal waters. Mar.Ecol.Prog.Ser. 417, 287-300.

Kortenkamp, A., Altenburger, R., 2010. Toxicity from combined exposure to chemicals. in: van Gestel, C., Jonker, M.J., Kammenga, J.E., Laskowski, R., Svendsen, C. (Eds.). Mixture Toxicity. Linking Approaches from Ecological and Human Toxicology. CRC Press. Taylor & Francis Group, Florida, pp. 95-119.

Lindeboom, H., 1984. The nitrogen pathway in a penguin rookery. Ecology 65, 269-277.

Liu, X., Zhao, S., Sun, L., Yin, X., Xie, Z., Honghao, L., Wang, Y., 2006. P and trace metal contents in biomaterials, soils, sediments and plants in colony of red-footed booby (*Sula sula*) in the Dongdao Island of South China Sea. Chemosphere 65, 707-715.

Long, E.R., Macdonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environ.Manage. 19, 81.

Lorenzo, J.I., Nieto, O., Beiras, R., 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquat.Toxicol. 58, 27-41.

623 Lorenzo, J.I., Nieto, O., Beiras, R., 2006. Anodic stripping voltammetry measures
624 copper bioavailability for sea urchin larvae in the presence of fulvic acids.
625 Environ.Toxicol.Chem. 25, 36-44.
626 Losso, C., Novelli, A.A., Picone, M., Ghetti, P.F., Ghirardini, A.V., 2009. Porewater as
627 a matrix in toxicity bioassays with sea urchins and bivalves: evaluation of applicability
628 to the Venice lagoon (Italy). Environ Int 35, 118-126.
629 Luis, O., Delgado, F., Gago, J., 2005. Year-round captive spawning performance of the
630 sea urchin *Paracentrotus lividus*: Relevance for the use of its larvae as live feed.
631 Aquat.Living Resour. 18, 45-54.
632 Markich, S.J., Brown, P.L., Jeffree, R.A., 2001. Divalent metal accumulation in
633 freshwater bivalves: an inverse relationship with metal phosphate solubility. Sci.Total
634 Environ. 275, 27-41.
635 Meyer, J.S., Farley, K.J., Garman, E.R., 2015. Metal mixtures modeling evaluation
636 project: 1. Background. Environ.Toxicol.Chem. 34, 726-740.
637 Michelutti, N., Blais, J.M., Mallory, M.L., Brash, J., Thienpont, J., Kimpe, L.E.,
638 Douglas, M.S., Smol, J.P., 2010. Trophic position influences the efficacy of seabirds as
639 metal biovectors. Proc.Natl.Acad.Sci.U.S.A. 107, 10543-10548.
640 Moawad, M.M., 2002. Complexation and thermal studies of uric acid with some
641 divalent and trivalent metal ions of biological interest in the solid state. J.Coord.Chem.
642 55, 61-78.
643 Motulsky, H.J., Christopoulos, A., 2003. Fitting models to biological data using linear
644 and nonlinear regression: a practical guide to curve fitting. Graphpad Software Inc., San
645 Diego CA.

646 Murado, M.A., Prieto, M.A., 2013. Dose-response analysis in the joint action of two
647 effectors. A new approach to simulation, identification and modelling of some basic
648 interactions. PLoS ONE 8, e61391 61391-61321.
649 Nadella, S.R., Tellis, M., Diamond, R., Smith, S., Bianchini, A., Wood, C.M., 2013.
650 Toxicity of lead and zinc to developing mussel and sea urchin embryos: critical tissue
651 residues and effects of dissolved organic matter and salinity. Comp.Biochem.Phys. C
652 158, 72-83.
653 OECD, 2000. Guidance document on aquatic toxicity testing of difficult substances and
654 mixtures. OECD Environmental Health and Safety Publications, Paris.
655 Otero, X.L., 1998. Effects of nesting yellow-legged gulls (*Larus cachinnans* Pallas) on
656 the heavy metal content of soils in the Cies Islands (Galicia, North-West Spain).
657 Mar.Pollut.Bull. 36, 267-272.
658 Otero, X.L., Fernandez-Sanjurjo, M.J., 2000. Mercury in faeces and feathers of yellow-
659 legged gulls (*Larus cachinnans*) and in soils from their breeding sites (Cies Islands-NW
660 Spain) in the vicinity of a chlor-alkali plant. Fresen.Environ.Bull. 9, 56-63.
661 Otero, X.L., Tejada, O., Martin-Pastor, M., De La Pena, S., Ferreira, T.O., Perez-
662 Alberti, A., 2015. Phosphorus in seagull colonies and the effect on the habitats. The
663 case of yellow-legged gulls (*Larus michahellis*) in the Atlantic Islands National Park
664 (Galicia-NW Spain). Sci.Total Environ. 532, 383-397.
665 Radenac, G., Fichet, D., Miramand, P., 2001. Bioaccumulation and toxicity of four
666 dissolved metals in *Paracentrotus lividus* sea-urchin embryo. Mar.Environ.Res. 51,
667 151-166.
668 Rial, D., Radovic, J.R., Bayona, J.M., Macrae, K., Thomas, K.V., Beiras, R., 2013.
669 Effects of simulated weathering on the toxicity of selected crude oils and their
670 components to sea urchin embryos. J.Hazard.Mater. 260, 67-73.

671 Saco-Álvarez, L., Durán, I., Lorenzo, J.I., Beiras, R., 2010. Methodological basis for the
672 optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of
673 coastal water quality. Ecotoxicol.Environ.Saf. 73, 491-499.
674 Saito, M.A., Moffett, J.W., 2001. Complexation of cobalt by natural organic ligands in
675 the Sargasso Sea as determined by a new high-sensitivity electrochemical cobalt
676 speciation method suitable for open ocean work. Mar.Chem. 75, 49-68.
677 Sánchez-Marín, P., Beiras, R., 2012. Quantification of the increase in Pb bioavailability
678 to marine organisms caused by different types of DOM from terrestrial and river origin.
679 Aquat.Toxicol. 110-111, 45-53.
680 Sanchez-Marin, P., Santos-Echeandia, J., Nieto-Cid, M., Alvarez-Salgado, X.A., Beiras,
681 R., 2010. Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb
682 speciation and toxicity to *Paracentrotus lividus* larvae. Aquat.Toxicol. 96, 90-102.
683 Santos-Echeandia, J., 2011. Direct simultaneous determination of Co, Cu, Fe, Ni and V
684 in pore waters by means of adsorptive cathodic stripping voltammetry with mixed
685 ligands. Talanta 85, 506-512.
686 Santos-Echeandia, J., Laglera, L.M., Prego, R., van den Berg, C.M.G., 2008. Dissolved
687 copper speciation behaviour during estuarine mixing in the San Simon Inlet (wet
688 season, Galicia). Influence of particulate matter. Estuar.Coast.Shelf S. 76, 447-453.
689 Signa, G., Tramati, C.D., Vizzini, S., 2013. Contamination by trace metals and their
690 trophic transfer to the biota in a Mediterranean coastal system affected by gull guano.
691 Mar.Ecol.Prog.Ser. 479, 13-24.
692 Smith, J.S., Johnson, C.R., 1995. Nutrient inputs from seabirds and humans on a
693 populated coral cay. Mar.Ecol.Prog.Ser. 124, 189-200.
694 Sovago, I., Kiss, T., Gergely, A., 1993. Critical survey of the stability constants of
695 complexes of aliphatic amino acids. Pure Appl.Chem. 65, 1029-1080.

696 Sun, L.G., Xie, Z.Q., Zhao, J.L., 2000. Palaeoecology - A 3,000-year record of penguin
697 populations. Nature 407, 858-858.
698 Szpak, P., Longstaffe, F.J., Millaire, J.F., White, C.D., 2012. Stable isotope
699 biogeochemistry of seabird guano fertilization: results from growth chamber studies
700 with maize (*Zea mays*). PLoS ONE 7, e33741.
701 Tak, H.K., Wilcox, W.R., Cooper, S.M., 1981. The effect of lead upon urate nucleation.
702 Arthritis Rheum. 24, 1291-1295.
703 Tellis, M.S., Lauer, M.M., Nadella, S., Bianchini, A., Wood, C.M., 2014a. The effects
704 of copper and nickel on the embryonic life stages of the purple sea urchin
705 (*Strongylocentrotus purpuratus*). Arch.Environ.Contam.Toxicol. 67, 453-464.
706 Tellis, M.S., Lauer, M.M., Nadella, S., Bianchini, A., Wood, C.M., 2014b. Sublethal
707 mechanisms of Pb and Zn toxicity to the purple sea urchin (*Strongylocentrotus*
708 *purpuratus*) during early development. Aquat.Toxicol. 146, 220-229.
709 Tovar-Sánchez, A., Basterretxea, G., Garcés, E., Masqué, P., García-Solsona, E.,
710 Marbà, N., 2011. Caracterización biogeoquímica del litoral del archipiélago de Cabrera.
711 Proyectos de investigación en parques nacionales: 2007-2010, pp. 33-45.
712 USEPA, 1996. Method 3052. Microwave assisted acid digestion of siliceous and
713 organically based matrices. p. 20.
714 van den Berg, C.M.G., Merks, A.G.A., Duursma, E.K., 1987. Organic complexation
715 and its control of the dissolved concentrations of copper and zinc in the Scheldt estuary.
716 Estuar.Coast.Shelf S. 24, 785-797.
717 Wait, D.A., Aubrey, D.P., Anderson, W.B., 2005. Seabird guano influences on desert
718 islands: soil chemistry and herbaceous species richness and productivity. J.Arid
719 Environ. 60, 681-695.

720 Wilcox, W.R., Weinberg.A, Klinenbe.Jr, Khalaf, A., Kippen, I., 1972. Solubility of
721 uric-acid and monosodium urate. Med.Biol.Eng. 10, 522-531.
722 Wootton, J.T., 1991. Direct and indirect effects of nutrients on intertidal community
723 structure - variable consequences of seabird guano. J.Exp.Mar.Biol.Ecol. 151, 139-153.
724 Xu, X., Li, Y., Wang, Y., Wang, Y., 2011. Assessment of toxic interactions of heavy
725 metals in multi-component mixtures using sea urchin embryo-larval bioassay. Toxicol.
726 In Vitro 25, 294-300.
727 Zelickman, E.A., Golovkin, A.N., 1972. Composition, structure and productivity of
728 neritic plankton communities near bird colonies of northern shores of Novaya Zemlya.
729 Mar.Biol. 17, 265-274.
730

731

732 **Figure captions**

733 Figure 1. Inhibition of sea urchin larval growth (R) by aqueous extracts of guano
734 obtained at different loads (left) and dissolved organic carbon measured on the extracts
735 (right). The symbols represent the loads of 0.462 g L^{-1} (○) and 1.952 g L^{-1} (●). The
736 lines represent the predictions of model 2 for the loads of 0.462 g L^{-1} (--), 1.952 g L^{-1}
737 (⋯) and all the results obtained (—). C_{guano}, concentration of guano in g/L. Error bars
738 are standard errors.

739

740 Figure 2. Inhibition of sea urchin larval growth (R) by Cu (left) and Pb (right) in
741 Artificial Sea Water (ASW) (—○—) and aqueous extracts of guano at low (--◇--) and
742 high concentration (⋯□⋯). The lines represent prediction of models 2 and 4 obtained
743 by individual fitting and the symbols represent the observed values. Concentrations of
744 Cu and Pb in μg L^{-1}. Error bars are standard errors.

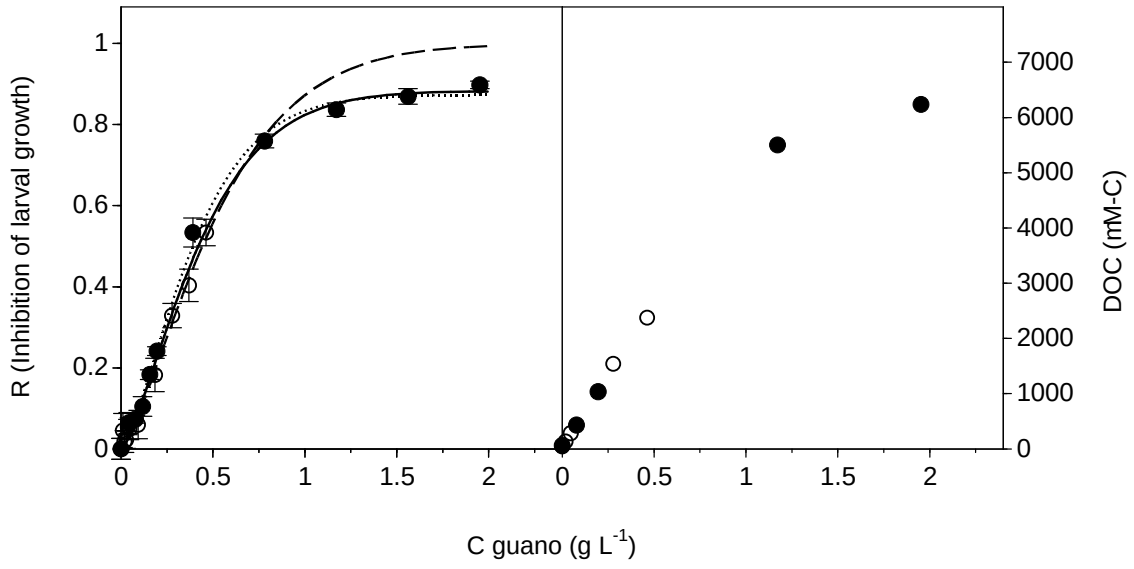
745

746 Figure 3. Inhibition of sea urchin larval growth (R) by joint action of Cu and Pb in
747 Artificial Sea Water (ASW) (up), aqueous extract of guano at low concentration
748 (middle) and aqueous extract of guano at high concentration (down). The symbols
749 represent the observed values and the response surface the prediction of model IA_SA
750 (12). Concentrations of Cu and Pb in μg L^{-1}.

751

752 **Figure 1**

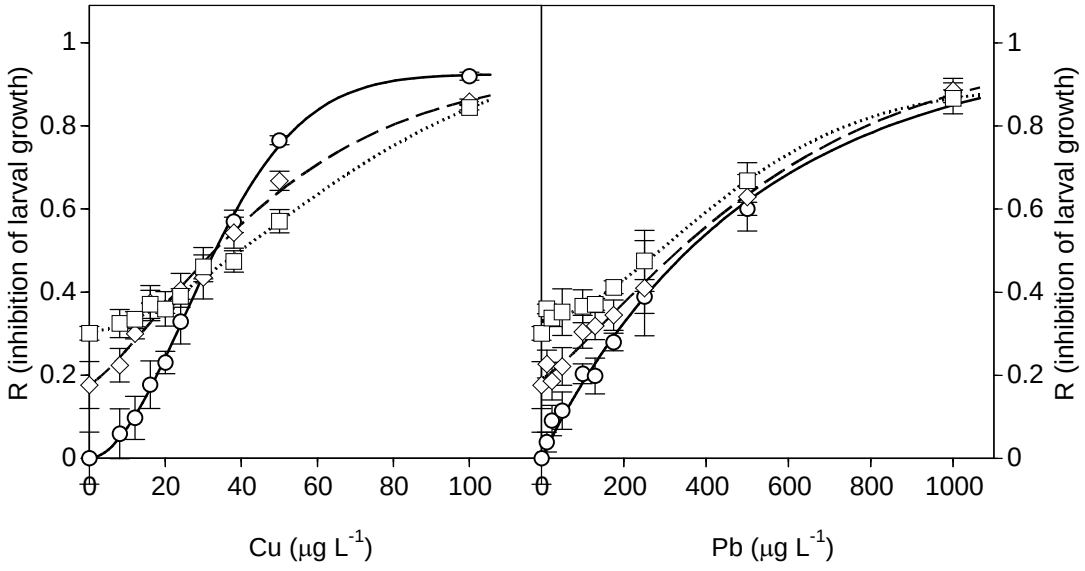
753



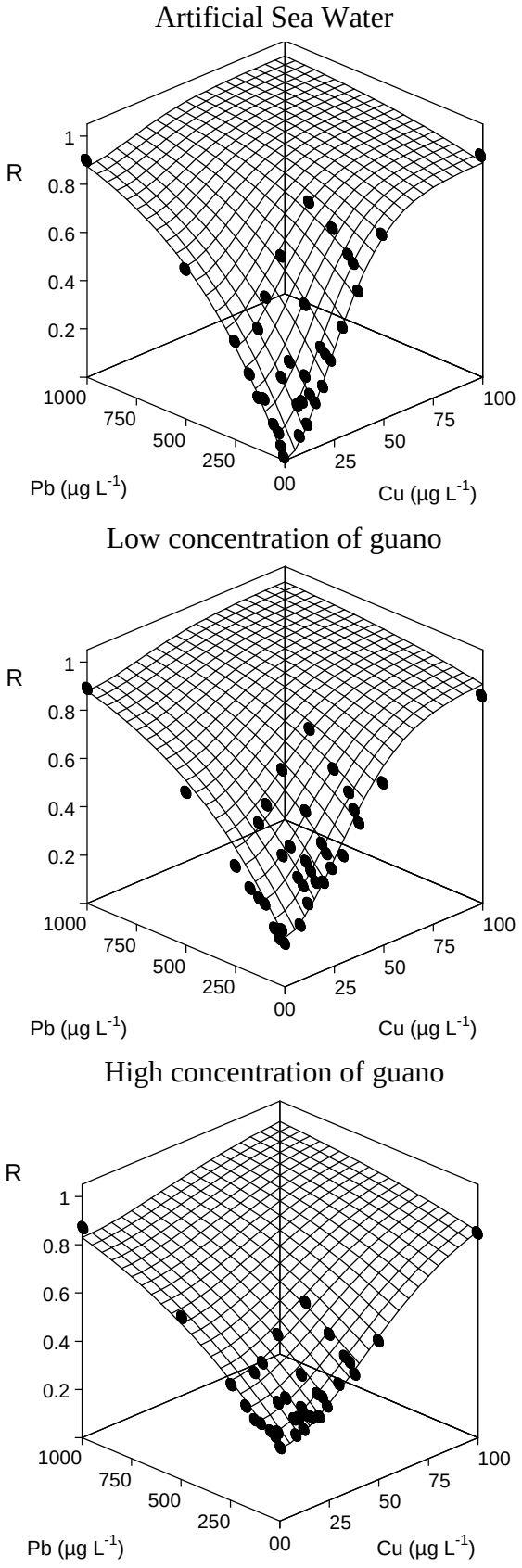
754

755

Figure 2


R (inhibition of larval growth)
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100
Cu (μg L-1)
0
200
400
600
800
1000
Pb (μg L-1)

759 **Figure 3**



760

Table captions

Table 1. Concentrations of dissolved organic carbon, nutrients and metals measured in the aqueous extracts obtained in the two tests performed.

Table 2. Summary of the parameters obtained by fitting model 2 to the toxicity results of the loading rate test. ns, not significant.

Table 3. Pb and Cu toxicity in: Artificial Sea Water (ASW), low concentration of extract of guano (Low guano) and high concentration of extract of guano (High guano). Summary of the parameters obtained by individual fitting of models 2, 3, 4 and 5 to Cu or Pb inhibition observed in the treatments indicated above.

Table 4. Parameters and goodness of fit of Concentration Addition (CA), Concentration Addition Simplified (CA_M), Concentration Addition Simplified adapted to describe Synergism or Antagonism (CA_SA), Independent Action (IA) and Independent Action Synergism or Antagonism (IA_SA). adj. R^2, adjusted R^2; AIC, Akaike Information Criterion; ns, not significant.

Table 1.

Experiment	Treatment	C_{guano} (g L^{-1})	DOC (µM-C)	NO_3^- (mM)	NO_2^- (mM)	NH_4^+ (mM)	PO_4^{3-} (mM)	SiO_2 (mM)	Cd (nM)	Cu (nM)	Pb (nM)	Ni (nM)	Co (nM)
Loading rate	Control	0	55	1.15	0.05	1.77	0.18	1.14	0.5	7.7	1.1	15.82	0.0655
	Low loading rate	0.01848	137	1.26	0.10	5.69	0.81	1.26					
		0.0462	285	1.20	0.18	11.40	1.99	1.05					
		0.2772	1543	0.84	0.82	48.66	10.60	1.64					
		0.462	2376	0.68	1.33	66.34	17.26	2.10	1.3	99.1	1.3	29.42	1.51
	High loading rate	0.07808	433	0.78	0.21	30.84	6.07	0.58					
		0.1952	1036	0.68	0.30	60.33	15.34	0.92					
		1.1712	5504		1.57	173.24	85.54	5.86					
		1.952	6236		2.58	168.70	102.60	10.75	4.0	104.9	2.9	31.66	2.32
Mixtures	Control	0	15	10.69	0.08	1.82	0.14	3.65	0.5	5.9	1.3	9.28	0.038
of Cu and Pb	Diluted aqueous extract of guano	0.015	149	2.72	0.16	8.46	1.73	4.04	0.6	23.9	1.8	10.16	0.115
	Concentrated aqueous extract of guano	0.073	375	0.29	0.58	31.24	8.39	4.33	0.8	79.6	5.4	11.59	0.295

Table 2.

Parameters	Low loading rate (0.462 g L^{-1})	High loading rate (1.952 g L^{-1})	Joint curve
K	1 ns	0.87±0.03	0.88±0.04
m (g L^{-1})		0.33±0.03	0.37±0.03
EC_{50} (g L^{-1})	0.44±0.03	0.39±0.03	0.42±0.03
EC_{10} (g L^{-1})	0.11±0.02	0.09±0.02	0.10±0.01
a	1.33±0.23	1.37±0.18	1.35±0.15

Table 3

Metal	Parameter	Treatment		
		ASW	Low Guano	High Guano
Cu	EC_{50} (μg L^{-1})	33.09±0.73	32.86±3.54	39.69±3.16
	a	1.87±0.12	1.29±0.55	1.63±0.70
	v_m (L μg^{-1})	0.0192±0.0013	0.0086±0.0037	0.0063±0.0025
Pb	EC_{50} μg L^{-1})	355.87±181.76	331.75±40.15	285.03±34.63
	a	0.98±0.23	1.24±0.44	1.69±0.54
	v_m (L μg^{-1})	0.0010±0.0004	0.0008±0.0003	0.0008±0.0002

Table 4

		Concentration Addition			Independent Action		
		Null interaction CA	Simplified CA_M	Antagonism CA_SA		Null interaction IA	Antagonism IA_SA
Cu	K	0.907±0.107	1.000±0.227	1.000±0.191	K_1	0.875±0.102	0.890±0.065
	m_1 (μg L^{-1})	32.2±4.8	36.9±13.8	31.6±9.4	m_1 (μg/L)	32.3±4.3	28.4±2.2
	a_1	1.703±0.447	0.931±0.195	1.065±0.195	a_1	1.575±0.272	1.807±0.199
	c_1 (L g^{-1})			12.162±7.570	b_{m1} (L/g)		11.580±3.162
Pb					K_2	1.000±0.376	1.000±0.220
	m_2 (μg L^{-1})	315.2±77.4			m_2 (μg/L)	376.6±248.8	352.7±131.1
	a_2	1.198±0.315			a_2	0.958±0.270	1.051±0.193
	u_2		0.106±0.018	0.083±0.015	b_{m2} (L/g)		5.974±3.977
Guano					K_3	0.396 ns	1.000 ns
	m_3 (g L^{-1})	0.7 ns			m_3 (g/L)	0.4 ns	0.8±0.8
	a_3	0.182 ns			a_3	0.044 ns	0.280±0.089
	u_3 (μg g^{-1})		131.863±1.846	175.631±42.103			
	adj. R^2	0.933	0.843	0.880		0.904	0.959
	AIC	-605.7	-544.0	-518.0		-564.5	-653.3